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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US90/04001 <b>(22) International Filing Date:</b> 17 July 1990 (17.07.90)  <b>(30) Priority data:</b> 403,241 5 September 1989 (05.09.89) US 405,370 11 September 1989 (11.09.89) US 421,417 13 October 1989 (13.10.89) US  <b>(71) Applicant:</b> IMMUNEX CORPORATION [US/US]; 51 University Street, Seattle, WA 98101 (US).  <b>(72) Inventors:</b> SMITH, Craig, A. ; 20405 5th West, Seattle, WA 98119 (US). GOODWIN, Raymond, G. ; 3322 8th Avenue West, Seattle, WA 98119 (US). BECKMANN, Patricia, M. ; 15875 Nesika Bay Road, Poulsbo, WA 98370 (US).	<b>(74) Agent:</b> WIGHT, Christopher, L.; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).  <b>(81) Designated States:</b> AU, CA, FI, KR, NO.  <b>Published</b> <i>With international search report.</i> <i>With amended claims and statement.</i>	
<b>(54) Title:</b> TUMOR NECROSIS FACTOR- $\alpha$ AND - $\beta$ RECEPTORS  <b>(57) Abstract</b>  Tumor necrosis factor receptor proteins, DNAs and expression vectors encoding TNF receptors, and processes for producing TNF receptors as products of recombinant cell culture, are disclosed.		

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**TITLE**Tumor Necrosis Factor- $\alpha$  and - $\beta$  Receptors

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**CROSS-REFERENCE TO RELATED APPLICATION**

This application is a continuation-in-part of U.S. application Serial No. 421,417, filed October 13, 1989, now pending, which is a continuation-in-part of U.S. application Serial No. 405,370, filed September 11, 1989, now abandoned, which is a continuation-in-part of  
10 U.S. application Serial No. 403,241, filed September 5, 1989, now abandoned.

**BACKGROUND OF THE INVENTION**

The present invention relates generally to cytokine receptors and more specifically to tumor necrosis factor receptors.

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Tumor necrosis factor- $\alpha$  (TNF $\alpha$ , also known as cachectin) and tumor necrosis factor- $\beta$  (TNF $\beta$ , also known as lymphotoxin) are homologous mammalian endogenous secretory proteins capable of inducing a wide variety of effects on a large number of cell types. The great similarities in the structural and functional characteristics of these two cytokines have resulted in their collective description as "TNF." Complementary cDNA clones encoding  
20 TNF $\alpha$  (Pennica et al., *Nature* 312:724, 1984) and TNF $\beta$  (Gray et al., *Nature* 312:721, 1984) have been isolated, permitting further structural and biological characterization of TNF.

TNF proteins initiate their biological effect on cells by binding to specific TNF receptor (TNF-R) proteins expressed on the plasma membrane of a TNF-responsive cell. TNF $\alpha$  and TNF $\beta$  were first shown to bind to a common receptor on the human cervical carcinoma cell line ME-180 (Aggarwal et al., *Nature* 318:665, 1985). Estimates of the size of the TNF-R determined by affinity labeling studies ranged from 54 to 175 kDa (Creasey et al.,  
25 *Proc. Natl. Acad. Sci. USA* 84:3293, 1987; Stauber et al., *J. Biol. Chem.* 263:19098, 1988; Hohmann et al., *J. Biol. Chem.* 264:14927, 1989). Although the relationship between these TNF-Rs of different molecular mass is unclear, Hohmann et al. (*J. Biol. Chem.* 264:14927,  
30 1989) reported that at least two different cell surface receptors for TNF exist on different cell types. These receptors have an apparent molecular mass of about 80 kDa and about 55-60 kDa, respectively. None of the above publications, however, reported the purification to homogeneity of cell surface TNF receptors.

In addition to cell surface receptors for TNF, soluble proteins from human urine  
35 capable of binding TNF have also been identified (Peetre et al., *Eur. J. Haematol.* 41:414, 1988; Seckinger et al., *J. Exp. Med.* 167:1511, 1988; Seckinger et al., *J. Biol. Chem.* 264:11966, 1989; UK Patent Application, Publ. No. 2 218 101 A to Seckinger et al.;

Engelmann et al., *J. Biol. Chem.* 264:11974, 1989). The soluble urinary TNF binding protein disclosed by UK 2 218 101 A has a partial N-terminal amino acid sequence of Asp-Ser-Val-Cys-Pro-, which corresponds to the partial sequence disclosed later by Engelmann et al. (1989). The relationship of the above soluble urinary binding proteins was further  
5 elucidated after original parent application (U.S. Serial No. 403,241) of the present application was filed, when Engelmann et al. reported the identification and purification of a second distinct soluble urinary TNF binding protein having an N-terminal amino acid sequence of Val-Ala-Phe-Thr-Pro- (*J. Biol. Chem.* 265:1531, 1990). The two urinary proteins disclosed by the UK 2 218 101 A and the Engelmann et al. publications were shown  
10 to be immunochemically related to two apparently distinct cell surface proteins by the ability of antiserum against the binding proteins to inhibit TNF binding to certain cells.

More recently, two separate groups reported the molecular cloning and expression of a human 55 kDa TNF-R (Loetscher et al., *Cell* 61:351, 1990; Schall et al., *Cell* 61:361, 1990). The TNF-R of both groups has an N-terminal amino acid sequence which  
15 corresponds to the partial amino acid sequence of the urinary binding protein disclosed by UK 2 218 101 A, Engelmann et al. (1989) and Engelmann et al. (1990).

In order to elucidate the relationship of the multiple forms of TNF-R and soluble urinary TNF binding proteins, or to study the structural and biological characteristics of TNF-Rs and the role played by TNF-Rs in the responses of various cell populations to TNF or  
20 other cytokine stimulation, or to use TNF-Rs effectively in therapy, diagnosis, or assay, purified compositions of TNF-R are needed. Such compositions, however, are obtainable in practical yields only by cloning and expressing genes encoding the receptors using recombinant DNA technology. Effort to purify the TNF-R molecule for use in biochemical analysis or to clone and express mammalian genes encoding TNF-R, however, have been  
25 impeded by lack of a suitable source of receptor protein or mRNA. Prior to the present invention, no cell lines were known to express high levels of TNF-R constitutively and continuously, which precluded purification of receptor for sequencing or construction of genetic libraries for cDNA cloning.

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### **SUMMARY OF THE INVENTION**

The present invention provides isolated TNF receptors and DNA sequences encoding mammalian tumor necrosis factor receptors (TNF-R), in particular, human TNF-Rs. Such DNA sequences include (a) cDNA clones having a nucleotide sequence derived from the coding region of a native TNF-R gene; (b) DNA sequences which are capable of  
35 hybridization to the cDNA clones of (a) under moderately stringent conditions and which encode biologically active TNF-R molecules; or (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode

biologically active TNF-R molecules. In particular, the present invention provides DNA sequences which encode soluble TNF receptors.

The present invention also provides recombinant expression vectors comprising the DNA sequences defined above, recombinant TNF-R molecules produced using the recombinant expression vectors, and processes for producing the recombinant TNF-R molecules using the expression vectors.

The present invention also provides isolated or purified protein compositions comprising TNF-R, and, in particular, soluble forms of TNF-R.

The present invention also provides compositions for use in therapy, diagnosis, assay of TNF-R, or in raising antibodies to TNF-R, comprising effective quantities of soluble native or recombinant receptor proteins prepared according to the foregoing processes.

Because of the ability of TNF to specifically bind TNF receptors (TNF-Rs), purified TNF-R compositions will be useful in diagnostic assays for TNF, as well as in raising antibodies to TNF receptor for use in diagnosis and therapy. In addition, purified TNF receptor compositions may be used directly in therapy to bind or scavenge TNF, thereby providing a means for regulating the immune activities of this cytokine.

These and other aspects of the present invention will become evident upon reference to the following detailed description.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a schematic representation of the coding region of various cDNAs encoding human and murine TNF-Rs. The leader sequence is hatched and the transmembrane region is solid.

Figures 2-3 depict the partial cDNA sequence and derived amino acid sequence of the human TNF-R clone 1. Nucleotides are numbered from the beginning of the 5' untranslated region. Amino acids are numbered from the beginning of the signal peptide sequence. The putative signal peptide sequence is represented by the amino acids -22 to -1. The N-terminal leucine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 236 to 265 is also underlined. The C-termini of various soluble TNF-Rs are marked with an arrow (↑).

Figures 4-6 depict the cDNA sequence and derived amino acid sequence of murine TNF-R clone 11. The putative signal peptide sequence is represented by amino acids -22 to -1. The N-terminal valine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 234 to 265 is also underlined.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **Definitions**

As used herein, the terms "TNF receptor" and "TNF-R" refer to proteins having amino acid sequences which are substantially similar to the native mammalian TNF receptor amino acid sequences, and which are biologically active, as defined below, in that they are capable of binding TNF molecules or transducing a biological signal initiated by a TNF molecule binding to a cell, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., nonrecombinant) sources. The mature full-length human TNF-R is a glycoprotein having a molecular weight of about 80 kilodaltons (kDa). As used throughout the specification, the term "mature" means a protein expressed in a form lacking a leader sequence as may be present in full-length transcripts of a native gene. Experiments using COS cells transfected with a cDNA encoding full-length human TNF-R showed that TNF-R bound  $^{125}\text{I}$ -TNF $\alpha$  with an apparent  $K_a$  of about  $5 \times 10^9 \text{ M}^{-1}$ , and that TNF-R bound  $^{125}\text{I}$ -TNF $\beta$  with an apparent  $K_a$  of about  $2 \times 10^9 \text{ M}^{-1}$ . The terms "TNF receptor" or "TNF-R" include, but are not limited to, analogs or subunits of native proteins having at least 20 amino acids and which exhibit at least some biological activity in common with TNF-R, for example, soluble TNF-R constructs which are devoid of a transmembrane region (and are secreted from the cell) but retain the ability to bind TNF. Various bioequivalent protein and amino acid analogs are described in detail below.

The nomenclature for TNF-R analogs as used herein follows the convention of naming the protein (e.g., TNF-R) preceded by either hu (for human) or mu (for murine) and followed by a  $\Delta$  (to designate a deletion) and the number of the C-terminal amino acid. For example, huTNF-R $\Delta$ 235 refers to human TNF-R having Asp<sup>235</sup> as the C-terminal amino acid (i.e., a polypeptide having the sequence of amino acids 1-235 of Figure 2). In the absence of any human or murine species designation, TNF-R refers generically to mammalian TNF-R. Similarly, in the absence of any specific designation for deletion mutants, the term TNF-R means all forms of TNF-R, including mutants and analogs which possess TNF-R biological activity.

"Soluble TNF-R" or "sTNF-R" as used in the context of the present invention refer to proteins, or substantially equivalent analogs, having an amino acid sequence corresponding to all or part of the extracellular region of a native TNF-R, for example, huTNF-R $\Delta$ 235, huTNF-R $\Delta$ 185 and huTNF-R $\Delta$ 163, or amino acid sequences substantially similar to the sequences of amino acids 1-163, amino acids 1-185, or amino acids 1-235 of Figure 2, and which are biologically active in that they bind to TNF ligand. Equivalent soluble TNF-Rs include polypeptides which vary from these sequences by one or more substitutions, deletions, or additions, and which retain the ability to bind TNF or inhibit TNF signal transduction activity via cell surface bound TNF receptor proteins, for example huTNF-R $\Delta$ x,

wherein x is selected from the group consisting of any one of amino acids 163-235 of Figure 2. Analogous deletions may be made to muTNF-R. Inhibition of TNF signal transduction activity can be determined by transfecting cells with recombinant TNF-R DNAs to obtain recombinant receptor expression. The cells are then contacted with TNF and the resulting metabolic effects examined. If an effect results which is attributable to the action of the ligand, then the recombinant receptor has signal transduction activity. Exemplary procedures for determining whether a polypeptide has signal transduction activity are disclosed by Idzerda et al., *J. Exp. Med.* 171:861 (1990); Curtis et al., *Proc. Natl. Acad. Sci. USA* 86:3045 (1989); Prywes et al., *EMBO J.* 5:2179 (1986) and Chou et al., *J. Biol. Chem.* 262:1842 (1987). Alternatively, primary cells or cell lines which express an endogenous TNF receptor and have a detectable biological response to TNF could also be utilized.

The term "isolated" or "purified", as used in the context of this specification to define the purity of TNF-R protein or protein compositions, means that the protein or protein composition is substantially free of other proteins of natural or endogenous origin and contains less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. TNF-R is isolated if it is detectable as a single protein band in a polyacrylamide gel by silver staining.

The term "substantially similar," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which is to retain biological activity of the TNF-R protein as may be determined, for example, in one of the TNF-R binding assays set forth in Example 1 below. Alternatively, nucleic acid subunits and analogs are "substantially similar" to the specific DNA sequences disclosed herein if: (a) the DNA sequence is derived from the coding region of a native mammalian TNF-R gene; (b) the DNA sequence is capable of hybridization to DNA sequences of (a) under moderately stringent conditions (50°C, 2x SSC) and which encode biologically active TNF-R molecules; or DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b) and which encode biologically active TNF-R molecules.

"Recombinant," as used herein, means that a protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.



"Biologically active," as used throughout the specification as a characteristic of TNF receptors, means that a particular molecule shares sufficient amino acid sequence similarity with the embodiments of the present invention disclosed herein to be capable of binding detectable quantities of TNF, transmitting a TNF stimulus to a cell, for example, as a component of a hybrid receptor construct, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., nonrecombinant) sources. Preferably, biologically active TNF receptors within the scope of the present invention are capable of binding greater than 0.1 nmoles TNF per nmole receptor, and most preferably, greater than 0.5 nmole TNF per nmole receptor in standard binding assays (see below).

"Isolated DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used as a source of coding sequences. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit.

#### Isolation of cDNAs Encoding TNF-R

The coding sequence of TNF-R is obtained by isolating a complementary DNA (cDNA) sequence encoding TNF-R from a recombinant cDNA or genomic DNA library. A cDNA library is preferably constructed by obtaining polyadenylated mRNA from a particular cell line which expresses a mammalian TNF-R, for example, the human fibroblast cell line WI-26 VA4 (ATCC CCL 95.1) and using the mRNA as a template for synthesizing double stranded cDNA. The double stranded cDNA is then packaged into a recombinant vector, which is introduced into an appropriate *E. coli* strain and propagated. Murine or other mammalian cell lines which express TNF-R may also be used. TNF-R sequences contained in the cDNA library can be readily identified by screening the library with an appropriate nucleic acid probe which is capable of hybridizing with TNF-R cDNA. Alternatively, DNAs encoding TNF-R proteins can be assembled by ligation of synthetic oligonucleotide subunits

corresponding to all or part of the sequence of Figures 2-3 or Figures 4-6 to provide a complete coding sequence.

The human TNF receptor cDNAs of the present invention were isolated by the method of direct expression cloning. A cDNA library was constructed by first isolating cytoplasmic mRNA from the human fibroblast cell line WI-26 VA4. Polyadenylated RNA was isolated and used to prepare double-stranded cDNA. Purified cDNA fragments were then ligated into pCAV/NOT vector DNA which uses regulatory sequences derived from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312:768, 1984), SV40 and cytomegalovirus DNA, described in detail below in Example 2. pCAV/NOT has been deposited with the American Type Culture Collection under accession No. ATCC 68014. The pCAV/NOT vectors containing the WI26-VA4 cDNA fragments were transformed into *E. coli* strain DH5 $\alpha$ . Transformants were plated to provide approximately 800 colonies per plate. The resulting colonies were harvested and each pool used to prepare plasmid DNA for transfection into COS-7 cells essentially as described by Cosman et al. (*Nature* 312:768, 1984) and Luthman et al. (*Nucl. Acid Res.* 11:1295, 1983). Transformants expressing biologically active cell surface TNF receptors were identified by screening for their ability to bind <sup>125</sup>I-TNF. In this screening approach, transfected COS-7 cells were incubated with medium containing <sup>125</sup>I-TNF, the cells washed to remove unbound labeled TNF, and the cell monolayers contacted with X-ray film to detect concentrations of TNF binding, as disclosed by Sims et al, *Science* 241:585 (1988). Transfectants detected in this manner appear as dark foci against a relatively light background.

Using this approach, approximately 240,000 cDNAs were screened in pools of approximately 800 cDNAs until assay of one transfectant pool indicated positive foci for TNF binding. A frozen stock of bacteria from this positive pool was grown in culture and plated to provide individual colonies, which were screened until a single clone (clone 11) was identified which was capable of directing synthesis of a surface protein with detectable TNF binding activity. The sequence of cDNA clone 11 isolated by the above method is depicted in Figures 4-6.

Additional cDNA clones can be isolated from cDNA libraries of other mammalian species by cross-species hybridization. For use in hybridization, DNA encoding TNF-R may be covalently labeled with a detectable substance such as a fluorescent group, a radioactive atom or a chemiluminescent group by methods well known to those skilled in the art. Such probes could also be used for *in vitro* diagnosis of particular conditions.

Like most mammalian genes, mammalian TNF receptors are presumably encoded by multi-exon genes. Alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share large regions of identity or similarity

with the cDNAs claimed herein, are considered to be within the scope of the present invention.

Other mammalian TNF-R cDNAs are isolated by using an appropriate human TNF-R DNA sequence as a probe for screening a particular mammalian cDNA library by cross-species hybridization.

### Proteins and Analogs

The present invention provides isolated recombinant mammalian TNF-R polypeptides. Isolated TNF-R polypeptides of this invention are substantially free of other contaminating materials of natural or endogenous origin and contain less than about 1% by mass of protein contaminants residual of production processes. The native human TNF-R molecules are recovered from cell lysates as glycoproteins having an apparent molecular weight by SDS-PAGE of about 80 kilodaltons (kDa). The TNF-R polypeptides of this invention are optionally without associated native-pattern glycosylation.

Mammalian TNF-R of the present invention includes, by way of example, primate, human, murine, canine, feline, bovine, ovine, equine and porcine TNF-R. Mammalian TNF-Rs can be obtained by cross species hybridization, using a single stranded cDNA derived from the human TNF-R DNA sequence as a hybridization probe to isolate TNF-R cDNAs from mammalian cDNA libraries.

Derivatives of TNF-R within the scope of the invention also include various structural forms of the primary protein which retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a TNF-R protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to TNF-R amino acid side chains or at the N- or C-termini. Other derivatives of TNF-R within the scope of this invention include covalent or aggregative conjugates of TNF-R or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast  $\alpha$ -factor leader). TNF-R protein fusions can comprise peptides added to facilitate purification or identification of TNF-R (e.g., poly-His). The amino acid sequence of TNF receptor can also be linked to the peptide Asp-Tyr-Lys-Asp-

Asp-Asp-Asp-Lys (DYKDDDDK) (Hopp et al., *Bio/Technology* 6:1204,1988.) The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*.

TNF-R derivatives may also be used as immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of TNF or other binding ligands. TNF-R derivatives may also be obtained by cross-linking agents, such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. TNF-R proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, TNF-R may be used to selectively bind (for purposes of assay or purification) anti-TNF-R antibodies or TNF.

The present invention also includes TNF-R with or without associated native-pattern glycosylation. TNF-R expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of TNF-R DNAs in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of mammalian TNF-R having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-A<sub>1</sub>-Z, where A<sub>1</sub> is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A<sub>1</sub> and Z, or an amino acid other than Asn between Asn and A<sub>1</sub>.

TNF-R derivatives may also be obtained by mutations of TNF-R or its subunits. A TNF-R mutant, as referred to herein, is a polypeptide homologous to TNF-R but which has an amino acid sequence different from native TNF-R because of a deletion, insertion or substitution.

Bioequivalent analogs of TNF-R proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted

(e.g., Cys<sup>178</sup>) or replaced with other amino acids to prevent formation of unnecessary or incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those having physiochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered. Substantially similar polypeptide sequences, as defined above, generally comprise a like number of amino acid sequences, although C-terminal truncations for the purpose of constructing soluble TNF-Rs will contain fewer amino acid sequences. In order to preserve the biological activity of TNF-Rs, deletions and substitutions will preferably result in homologous or conservatively substituted sequences, meaning that a given residue is replaced by a biologically similar residue. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Moreover, particular amino acid differences between human, murine and other mammalian TNF-Rs is suggestive of additional conservative substitutions that may be made without altering the essential biological characteristics of TNF-R.

Subunits of TNF-R may be constructed by deleting terminal or internal residues or sequences. Particularly preferred sequences include those in which the transmembrane region and intracellular domain of TNF-R are deleted or substituted with hydrophilic residues to facilitate secretion of the receptor into the cell culture medium. The resulting protein is referred to as a soluble TNF-R molecule which retains its ability to bind TNF. A particularly preferred soluble TNF-R construct is TNF-RA235 (the sequence of amino acids 1-235 of Figure 2), which comprises the entire extracellular region of TNF-R, terminating with Asp<sup>235</sup> immediately adjacent the transmembrane region. Additional amino acids may be deleted from the transmembrane region while retaining TNF binding activity. For example, huTNF-RA183 which comprises the sequence of amino acids 1-183 of Figure 2, and TNF-RA163 which comprises the sequence of amino acids 1-163 of Figure 2, retain the ability to bind TNF ligand as determined using the binding assays described below in Example 1. TNF-RA142, however, does not retain the ability to bind TNF ligand. This suggests that one or both of Cys<sup>157</sup> and Cys<sup>163</sup> is required for formation of an intramolecular disulfide bridge for the proper folding of TNF-R. Cys<sup>178</sup>, which was deleted without any apparent adverse effect on the ability of the soluble TNF-R to bind TNF, does not appear to be essential for

proper folding of TNF-R. Thus, any deletion C-terminal to Cys<sup>163</sup> would be expected to result in a biologically active soluble TNF-R. The present invention contemplates such soluble TNF-R constructs corresponding to all or part of the extracellular region of TNF-R terminating with any amino acid after Cys<sup>163</sup>. Other C-terminal deletions, such as TNF-  
5 FA157, may be made as a matter of convenience by cutting TNF-R cDNA with appropriate restriction enzymes and, if necessary, reconstructing specific sequences with synthetic oligonucleotide linkers. The resulting soluble TNF-R constructs are then inserted and expressed in appropriate expression vectors and assayed for the ability to bind TNF, as described in Example 1. Biologically active soluble TNF-Rs resulting from such  
10 constructions are also contemplated to be within the scope of the present invention.

Mutations in nucleotide sequences constructed for expression of analog TNF-R must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the receptor mRNA.  
15 Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed TNF-R mutants screened for the desired activity.

Not all mutations in the nucleotide sequence which encodes TNF-R will be expressed  
20 in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

25 Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be  
30 employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and  
35 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Both mon valent forms and polyvalent forms of TNF-R are useful in the compositions and methods of this invention. Polyvalent forms possess multiple TNF-R

binding sites for TNF ligand. For example, a bivalent soluble TNF-R may consist of two tandem repeats of amino acids 1-235 of Figure 2, separated by a linker region. Alternate polyvalent forms may also be constructed, for example, by chemically coupling TNF-R to any clinically acceptable carrier molecule, a polymer selected from the group consisting of

5 Ficoll, polyethylene glycol or dextran using conventional coupling techniques. Alternatively, TNF-R may be chemically coupled to biotin, and the biotin-TNF-R conjugate then allowed to bind to avidin, resulting in tetravalent avidin/biotin/TNF-R molecules. TNF-R may also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugate precipitated with anti-DNP or anti-TNP-IgM, to form decameric conjugates with a

10 valency of 10 for TNF-R binding sites.

A recombinant chimeric antibody molecule may also be produced having TNF-R sequences substituted for the variable domains of either or both of the immunoglobulin molecule heavy and light chains and having unmodified constant region domains. For example, chimeric TNF-R/IgG<sub>1</sub> may be produced from two chimeric genes -- a TNF-

15 R/human  $\kappa$  light chain chimera (TNF-R/C $\kappa$ ) and a TNF-R/human  $\gamma$ <sub>1</sub> heavy chain chimera (TNF-R/C $\gamma$ <sub>1</sub>). Following transcription and translation of the two chimeric genes, the gene products assemble into a single chimeric antibody molecule having TNF-R displayed bivalently. Such polyvalent forms of TNF-R may have enhanced binding affinity for TNF ligand. Additional details relating to the construction of such chimeric antibody molecules are

20 disclosed in WO 89/09622 and EP 315062.

#### Expression of Recombinant TNF-R

The present invention provides recombinant expression vectors to amplify or express DNA encoding TNF-R. Recombinant expression vectors are replicable DNA constructs

25 which have synthetic or cDNA-derived DNA fragments encoding mammalian TNF-R or bioequivalent analogs operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or

30 coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements may include an operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to

35 facilitate recognition of transformants may additionally be incorporated. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed

as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory  
5 leaders, contiguous and in reading frame. Structural elements intended for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to  
10 provide a final product.

DNA sequences encoding mammalian TNF receptors which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA; however, premature termination of transcription may be desirable, for example, where it would result in mutants having advantageous C-terminal  
15 truncations, for example, deletion of a transmembrane region to yield a soluble receptor not bound to the cell membrane. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing to the sequences of the provided cDNA under moderately stringent conditions (50°C, 2x SSC) and other sequences hybridizing or degenerate to those  
20 which encode biologically active TNF receptor polypeptides.

Recombinant TNF-R DNA is expressed or amplified in a recombinant expression system comprising a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated (by transformation or transfection) a recombinant transcriptional unit  
25 into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

30 Transformed host cells are cells which have been transformed or transfected with TNF-R vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express TNF-R, but host cells transformed for purposes of cloning or amplifying TNF-R DNA do not need to express TNF-R. Expressed TNF-R will be deposited in the cell membrane or secreted into the culture supernatant, depending on the TNF-R DNA selected.  
35 Suitable host cells for expression of mammalian TNF-R include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells



include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce mammalian TNF-R using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. 5 (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of TNF-R that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding 10 proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

15 Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" 20 sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

25 Promoters commonly used in recombinant microbial expression vectors include the  $\beta$ -lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A 30 particularly useful bacterial expression system employs the phage  $\lambda$  P<sub>L</sub> promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the  $\lambda$  P<sub>L</sub> promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Recombinant TNF-R proteins may also be expressed in yeast hosts, preferably from 35 the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2 $\mu$  yeast plasmid or an autonomously replicating sequence (ARS).

promoter, DNA encoding TNF-R, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 or URA3 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the TRP1 or URA3 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan or uracil.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pUC18 for selection and replication in *E. coli* (Amp<sup>r</sup> gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and  $\alpha$ -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast  $\alpha$ -factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. See, e.g., Kurjan et al., *Cell* 30:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978, selecting for Trp<sup>+</sup> transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10  $\mu$ g/ml adenine and 20  $\mu$ g/ml uracil or URA<sup>+</sup> transformants in medium consisting of 0.67% YNB, with amino acids and bases as described by Sherman et al., *Laboratory Course Manual for Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1986.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% or 4% glucose supplemented with 80  $\mu$ g/ml adenine and 80  $\mu$ g/ml uracil. Derepression of the ADH2

promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells is particularly preferred because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' untranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *BioTechnology* 6:47 (1988).

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* 3 site toward the *Bgl*1 site located in the viral origin of replication is included. Further, mammalian genomic TNF-R promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Additional details regarding the use of a mammalian high expression vector to produce a recombinant mammalian TNF receptor are provided in Examples 2 and 7 below. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986).

In preferred aspects of the present invention, recombinant expression vectors comprising TNF-R cDNAs are stably integrated into a host cell's DNA. Elevated levels of expression product is achieved by selecting for cell lines having amplified numbers of vector

DNA. Cell lines having amplified numbers of vector DNA are selected, for example, by transforming a host cell with a vector comprising a DNA sequence which encodes an enzyme which is inhibited by a known drug. The vector may also comprise a DNA sequence which encodes a desired protein. Alternatively, the host cell may be co-transformed with a second  
5 vector which comprises the DNA sequence which encodes the desired protein. The transformed or co-transformed host cells are then cultured in increasing concentrations of the known drug, thereby selecting for drug-resistant cells. Such drug-resistant cells survive in increased concentrations of the toxic drug by over-production of the enzyme which is inhibited by the drug, frequently as a result of amplification of the gene encoding the enzyme.  
10 Where drug resistance is caused by an increase in the copy number of the vector DNA encoding the inhibitable enzyme, there is a concomitant co-amplification of the vector DNA encoding the desired protein (TNF-R) in the host cell's DNA.

A preferred system for such co-amplification uses the gene for dihydrofolate reductase (DHFR), which can be inhibited by the drug methotrexate (MTX). To achieve co-  
15 amplification, a host cell which lacks an active gene encoding DHFR is either transformed with a vector which comprises DNA sequence encoding DHFR and a desired protein, or is co-transformed with a vector comprising a DNA sequence encoding DHFR and a vector comprising a DNA sequence encoding the desired protein. The transformed or co-transformed host cells are cultured in media containing increasing levels of MTX, and those  
20 cells lines which survive are selected.

A particularly preferred co-amplification system uses the gene for glutamine synthetase (GS), which is responsible for the synthesis of glutamate and ammonia using the hydrolysis of ATP to ADP and phosphate to drive the reaction. GS is subject to inhibition by a variety of inhibitors, for example methionine sulfoximine (MSX). Thus, TNF-R can be  
25 expressed in high concentrations by co-amplifying cells transformed with a vector comprising the DNA sequence for GS and a desired protein, or co-transformed with a vector comprising a DNA sequence encoding GS and a vector comprising a DNA sequence encoding the desired protein, culturing the host cells in media containing increasing levels of MSX and selecting for surviving cells. The GS co-amplification system, appropriate recombinant expression  
30 vectors and cells lines, are described in the following PCT applications: WO 87/04462, WO 89/01036, WO 89/10404 and WO 86/05807.

Recombinant proteins are preferably expressed by co-amplification of DHFR or GS in a mammalian host cell, such as Chinese Hamster Ovary (CHO) cells, or alternatively in a murine myeloma cell line, such as SP2/0-Ag14 or NS0 or a rat myeloma cell line, such as  
35 YB2/3.0-Ag20, disclosed in PCT applications WO/89/10404 and WO 86/05807.

A preferred eukaryotic vector for expression of TNF-R DNA is disclosed below in Example 2. This vector, referred to as pCAV/NOT, was derived from the mammalian high

expression vector pDC201 and contains regulatory sequences from SV40, adenovirus-2, and human cytomegalovirus.

#### Purification of Recombinant TNF-R

5        Purified mammalian TNF receptors or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts.

10        For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a TNF or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be  
15        acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

20        Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a TNF-R composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

25        Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant mammalian TNF-R can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use  
30        of cell lysing agents.

35        Fermentation of yeast which express mammalian TNF-R as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Human TNF-R synthesized in recombinant culture is characterized by the presence of non-human cell components, including proteins, in amounts and of a character which depend

upon the purification steps taken to recover human TNF-R from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of TNF-R free of proteins which may be normally associated with TNF-R as it is found in nature in its species of origin, e.g. in cells, cell exudates or body fluids.

#### Therapeutic Administration of Recombinant Soluble TNF-R

The present invention provides methods of using therapeutic compositions comprising an effective amount of soluble TNF-R proteins and a suitable diluent and carrier, and methods for suppressing TNF-dependent inflammatory responses in humans comprising administering an effective amount of soluble TNF-R protein.

For therapeutic use, purified soluble TNF-R protein is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, soluble TNF-R protein compositions can be administered by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a soluble TNF-R therapeutic agent will be administered in the form of a composition comprising purified protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNF-R with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

Soluble TNF-R proteins are administered for the purpose of inhibiting TNF-dependent responses. A variety of diseases or conditions are believed to be caused by TNF, such as cachexia and septic shock. In addition, other key cytokines (IL-1, IL-2 and other colony stimulating factors) can also induce significant host production of TNF. Soluble TNF-R compositions may therefore be used, for example, to treat cachexia or septic shock or to treat side effects associated with cytokine therapy. Because of the primary roles IL-1 and IL-2 play in the production of TNF, combination therapy using both IL-1 receptors or IL-2 receptors may be preferred in the treatment of TNF-associated clinical indications.

The following examples are offered by way of illustration, and not by way of limitation.

5

## EXAMPLES

### Example 1

#### Binding Assays

10

A. *Radiolabeling of TNF $\alpha$  and TNF $\beta$* . Recombinant human TNF $\alpha$ , in the form of a fusion protein containing a hydrophilic octapeptide at the N-terminus, was expressed in yeast as a secreted protein and purified by affinity chromatography (Hopp et al., *BioTechnology* 6:1204, 1988). Purified recombinant human TNF $\beta$  was purchased from R&D Systems (Minneapolis, MN). Both proteins were radiolabeled using the commercially available solid phase agent, IODO-GEN (Pierce). In this procedure, 5  $\mu$ g of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for 20 minutes at 4°C with 75  $\mu$ l of 0.1 M sodium phosphate, pH 7.4 and 20  $\mu$ l (2 mCi) Na <sup>125</sup>I. This solution was then transferred to a second glass tube containing 5  $\mu$ g TNF $\alpha$  (or TNF $\beta$ ) in 45  $\mu$ l PBS for 20 minutes at 4°C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex G-25 (Sigma) equilibrated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium azide and 20 mM Hepes pH 7.4 (binding medium). The final pool of <sup>125</sup>I-TNF was diluted to a working stock solution of 1 x 10<sup>-7</sup> M in binding medium and stored for up to one month at 4°C without detectable loss of receptor binding activity. The specific activity is routinely 1 x 10<sup>6</sup> cpm/mmol TNF.

B. *Binding to Intact Cells*. Binding assays with intact cells were performed by two methods. In the first method, cells were first grown either in suspension (e.g., U 937) or by adherence on tissue culture plates (e.g., WI26-VA4, COS cells expressing the recombinant TNF receptor). Adherent cells were subsequently removed by treatment with 5mM EDTA treatment for ten minutes at 37 degrees centigrade. Binding assays were then performed by a phthalate oil separation method (Dower et al., *J. Immunol.* 132:751, 1984) essentially as described by Park et al. (*J. Biol. Chem.* 261:4177, 1986). Non-specific binding of <sup>125</sup>I-TNF was measured in the presence of a 200-fold or greater molar excess of unlabeled TNF. Sodium azide (0.2%) was included in a binding assay to inhibit internalization of <sup>125</sup>I-TNF by cells. In the second method, COS cells transfected with the TNF-R-containing plasmid, and expressing TNF receptors on the surface, were tested for the ability to bind <sup>125</sup>I-TNF by the plate binding assay described by Sims et al. (*Science* 241:585, 1988).

35

C. *Solid Phase Binding Assays*. The ability of TNF-R to be stably adsorbed to nitrocellulose from detergent extracts of human cells yet retain TNF-binding activity provided

a means of detecting TNF-R. Cell extracts were prepared by mixing a cell pellet with a 2 x volume of PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10  $\mu$ M pepstatin, 10  $\mu$ M leupeptin, 2 mM o-phenanthroline and 2 mM EGTA) by vigorous vortexing. The mixture was incubated on ice for 30 minutes after which it was centrifuged at 12,000x g for 15 minutes at 8°C to remove nuclei and other debris. Two microliter aliquots of cell extracts were placed on dry BA85/21 nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and allowed to dry. The membranes were incubated in tissue culture dishes for 30 minutes in Tris (0.05 M) buffered saline (0.15 M) pH 7.5 containing 3% w/v BSA to block nonspecific binding sites. The membrane was then covered with  $5 \times 10^{-11}$  M  $^{125}$ I-TNF in PBS + 3% BSA and incubated for 2 hr at 4°C with shaking. At the end of this time, the membranes were washed 3 times in PBS, dried and placed on Kodak X-Omat AR film for 18 hr at -70°C.

## Example 2

### 15 Isolation of Human TNF-R cDNA by Direct Expression of Active Protein in COS-7 Cells

Various human cell lines were screened for expression of TNF-R based on their ability to bind  $^{125}$ I-labeled TNF. The human fibroblast cell line WI-26 VA4 was found to express a reasonable number of receptors per cell. Equilibrium binding studies showed that the cell line exhibited biphasic binding of  $^{125}$ I-TNF with approximately 4,000 high affinity sites ( $K_a = 1 \times 10^{10}$  M $^{-1}$ ) and 15,00 low affinity sites ( $K_a = 1 \times 10^8$  M $^{-1}$ ) per cell.

An unsized cDNA library was constructed by reverse transcription of polyadenylated mRNA isolated from total RNA extracted from human fibroblast WI-26 VA4 cells grown in the presence of pokeweed mitogen using standard techniques (Gubler, et al., *Gene* 25:263, 1983; Ausubel et al., eds., *Current Protocols in Molecular Biology*, Vol. 1, 1987). The cells were harvested by lysing the cells in a guanidine hydrochloride solution and total RNA isolated as previously described (March et al., *Nature* 315:641, 1985).

Poly A<sup>+</sup> RNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was prepared by a method similar to that of Gubler and Hoffman (*Gene* 25:263, 1983). Briefly, the poly A<sup>+</sup> RNA was converted to an RNA-cDNA hybrid by reverse transcriptase using oligo dT as a primer. The RNA-cDNA hybrid was then converted into double-stranded cDNA using RNAase H in combination with DNA polymerase I. The resulting double stranded cDNA was blunt-ended with T4 DNA polymerase. To the blunt-ended cDNA is added *Eco*RI linker-adapters (having internal *Not*I sites) which were phosphorylated on only one end (Invitrogen). The linker-adapted cDNA was treated with T4 polynucleotide kinase to phosphorylate the 5' overhanging region of the linker-adaptor and unligated linkers were removed by running the cDNA over a Sepharose CL4B column. The linker-adapted cDNA was ligated to an equimolar concentration of *Eco*RI cut and



dephosphorylated arms of bacteriophage  $\lambda$ gt10 (Huynh et al, *DNA Cloning: A Practical Approach*, Glover, ed., IRL Press, pp. 49-78). The ligated DNA was packaged into phage particles using a commercially available kit to generate a library of recombinants (Stratagene Cloning Systems, San Diego, CA, USA). Recombinants were further amplified by plating  
5 phage on a bacterial lawn of *E. coli* strain c600(hfl<sup>-</sup>).

Phage DNA was purified from the resulting  $\lambda$ gt10 cDNA library and the cDNA inserts excised by digestion with the restriction enzyme *Not*I. Following electrophoresis of the digest through an agarose gel, cDNAs greater than 2,000 bp were isolated.

The resulting cDNAs were ligated into the eukaryotic expression vector pCAV/NOT,  
10 which was designed to express cDNA sequences inserted at its multiple cloning site when transfected into mammalian cells. pCAV/NOT was assembled from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312: 768, 1984), SV40 and cytomegalovirus DNA and comprises, in sequence with the direction of transcription from the origin of replication: (1) SV40 sequences from coordinates 5171-270 including the origin of  
15 replication, enhancer sequences and early and late promoters; (2) cytomegalovirus sequences including the promoter and enhancer regions (nucleotides 671 to +63 from the sequence published by Boechart et al. (*Cell* 41:521, 1985); (3) adenovirus-2 sequences containing the first exon and part of the intron between the first and second exons of the tripartite leader, the second exon and part of the third exon of the tripartite leader and a multiple cloning site  
20 (MCS) containing sites for *Xho*I, *Kpn*I, *Sma*I, *Not*I and *Bgl*I; (4) SV40 sequences from coordinates 4127-4100 and 2770-2533 that include the polyadenylation and termination signals for early transcription; (5) sequences derived from pBR322 and virus-associated sequences VAI and VAII of pDC201, with adenovirus sequences 10532-11156 containing the VAI and VAII genes, followed by pBR322 sequences from 4363-2486 and 1094-375  
25 containing the ampicillin resistance gene and origin of replication.

The resulting WI-26 VA4 cDNA library in pCAV/NOT was used to transform *E. coli* strain DH5 $\alpha$ , and recombinants were plated to provide approximately 800 colonies per plate and sufficient plates to provide approximately 50,000 total colonies per screen. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The  
30 pooled DNA was then used to transfect a sub-confluent layer of monkey COS-7 cells using DEAE-dextran followed by chloroquine treatment, as described by Luthman et al. (*Nucl. Acids Res.* 11:1295, 1983) and McCutchan et al. (*J. Natl. Cancer Inst.* 41:351, 1986). The cells were then grown in culture for three days to permit transient expression of the inserted sequences. After three days, cell culture supernatants were discarded and the cell monolayers  
35 in each plate assayed for TNF binding as follows. Three ml of binding medium containing  $1.2 \times 10^{-11}$  M <sup>125</sup>I-labeled FLAG<sup>®</sup>-TNF was added to each plate and the plates incubated at 4°C for 120 minutes. This medium was then discarded, and each plate was washed once

with cold binding medium (containing no labeled TNF) and twice with cold PBS. The edges of each plate were then broken off, leaving a flat disk which was contacted with X-ray film for 72 hours at -70°C using an intensifying screen. TNF binding activity was visualized on the exposed films as a dark focus against a relatively uniform background.

5 After approximately 240,000 recombinants from the library had been screened in this manner, one transfectant pool was observed to provide TNF binding foci which were clearly apparent against the background exposure.

A frozen stock of bacteria from the positive pool was then used to obtain plates of approximately 150 colonies. Replicas of these plates were made on nitrocellulose filters, and the plates were then scraped and plasmid DNA prepared and transfected as described above to 10 identify a positive plate. Bacteria from individual colonies from the nitrocellulose replica of this plate were grown in 0.2 ml cultures, which were used to obtain plasmid DNA, which was transfected into COS-7 cells as described above. In this manner, a single clone, clone 1, was isolated which was capable of inducing expression of human TNF-R in COS cells. The 15 expression vector pCAV/NOT containing the TNF-R cDNA clone 1 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA (Accession No. 68088) under the name pCAV/NOT-TNF-R.

### Example 3

#### 20 Construction of cDNAs Encoding Soluble huTNF-RA235

A cDNA encoding a soluble huTNF-RA235 (having the sequence of amino acids 1-235 of Figure 2) was constructed by excising an 840 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Pvu2. Not1 cuts at the multiple cloning site of 25 pCAV/NOT-TNF-R and Pvu2 cuts within the TNF-R coding region 20 nucleotides 5' of the transmembrane region. In order to reconstruct the 3' end of the TNF-R sequences, two oligonucleotides were synthesized and annealed to create the following oligonucleotide linker:

30 Pvu2 BamH1 Bgl2  
CTGAAGGGAGCACTGGCGACTAAGGATCCA  
GACTTCCCTCGTGACCGCTGATTCCTAGGTCTAG  
AlaGluGlySerThrGlyAspEnd

This oligonucleotide linker has terminal Pvu2 and Bgl2 restriction sites, regenerates 20 35 nucleotides of the TNF-R, followed by a termination codon (underlined) and a BamH1 restriction site (for convenience in isolating the entire soluble TNF-R by Not1/BamH1 digestion). This oligonucleotide was then ligated with the 840 bp Not1/Pvu2 TNF-R insert into Bgl2/Not1 cut pCAV/NOT to yield psolhuTNF-RA235/CAVNOT, which was

transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R which was capable of binding TNF.

#### Example 4

##### 5                    Construction of cDNAs Encoding Soluble huTNF-RA185

A cDNA encoding a soluble huTNF-RA185 (having the sequence of amino acids 1-185 of Figure 2) was constructed by excising a 640 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Bgl2. Not1 cuts at the multiple cloning site of pCAV/NO-TNF-R and Bgl2 cuts within the TNF-R coding region at nucleotide 637, which is 10                    237 nucleotides 5' of the transmembrane region. The following oligonucleotide linkers were synthesized:

15                    Bgl2  
5'-GATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGCATGGATGC-3'  
                  ACATTGCACCACCGGTAGGGACCCTTACGTTTCG  
                  IleCysAsnValValAlaIleProGlyAsnAlaSerMetAspAla

20                    Not1  
5'-                  AGTCTGCACGTCCACGTCCCCACCCGGTGAGC                    -3'  
                  TACCTACGTGAGACGTGCAGGTGCAGGGGTGGGCCACTCGCCGG  
                  ValCysThrSerThrSerProThrArgEnd

25                    The above oligonucleotide linkers reconstruct the 3' end of the receptor molecule up to nucleotide 708, followed by a termination codon (underlined). These oligonucleotides were then ligated with the 640 bp Not1 TNF-R insert into Not1 cut pCAV/NOT to yield the expression vector psolTNFRA185/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R which 30                    was capable of binding TNF.

#### Example 5

##### Construction of cDNAs Encoding Soluble huTNF-RA163

35                    A cDNA encoding a soluble huTNF-RA163 (having the sequence of amino acids 1-163 of Figure 2) was constructed by excising a 640 bp fragment from from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Bgl2 as described in Example 4. The following oligonucleotide linkers were synthesized:

40                    Bgl2                    Not1  
5'-GATCTGTTGAGC                    -3'  
                  ACAACTCGCCGG  
                  IleCysEnd

This above oligonucleotide linker reconstructs the 3' end of the receptor molecule up to nucleotide 642 (amino acid 163), followed by a termination codon (underlined). This oligonucleotide was then ligated with the 640 bp Not1 TNF-R insert into Not1 cut pCAV/NOT to yield the expression vector psolTNFRA163/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R which was capable of binding TNF in the binding assay described in Example 1.

### Example 6

#### Construction of cDNAs Encoding Soluble huTNF-RΔ142

A cDNA encoding a soluble huTNF-RΔ142 (having the sequence of amino acids 1-142 of Figure 2) was constructed by excising a 550 bp fragment from from pCAV/NOT-TNF-R with the restriction enzymes Not1 and AlwN1. AlwN1 cuts within the TNF-R coding region at nucleotide 549. The following oligonucleotide linker was synthesized:

Bgl2                      Not1  
5' -CTGAAACATCAGACGTGGTGTGCAAGCCCTGTTAAA-3'  
CTTGACTTTGTAGTCTGCACCACACGTTCCGGGACAATTTCTAGA  
End

This above oligonucleotide linker reconstructs the 3' end of the receptor molecule up to nucleotide 579 (amino acid 142), followed by a termination codon (underlined). This oligonucleotide was then ligated with the 550 bp Not1/AlwN1 TNF-R insert into Not1/Bgl2 cut pCAV/NOT to yield the expression vector psolTNFRA142/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector did not induced expression of soluble human TNF-R which was capable of binding TNF. It is believed that this particular construct failed to express biologically active TNF-R because one or more essential cysteine residue (e.g., Cys<sup>157</sup> or Cys<sup>163</sup>) required for intramolecular bonding (for formation of the proper tertiary structure of the TNF-R molecule) was eliminated.

### Example 7

#### Expression of Soluble TNF Receptors in CHO Cells

Soluble TNF receptor was expressed in Chinese Hamster Ovary (CHO) cells using the glutamine-synthetase (GS) gene amplification system, substantially as described in PCT patent application Nos. WO87/04462 and WO89/01036. Briefly, CHO cells are transfected with an expression vector containing genes for both TNF-R and GS. CHO cells are selected

for GS gene expression based on the ability of the transfected DNA to confer resistance to low levels of methionine sulfoximine (MSX). GS sequence amplification events in such cells are selected using elevated MSX concentrations. In this way, contiguous TNF-R sequences are also amplified and enhanced TNF-R expression is achieved.

5       The vector used in the GS expression system was psolTNFR/P6/PSVLGS, which was constructed as follows. First, the vector pSVLGS.1 (described in PCT Application Nos. WO87/04462 and WO89/01036, and available from Celltech, Ltd., Berkshire, UK) was cut with the BamH1 restriction enzyme and dephosphorylated with calf intestinal alkaline phosphatase (CIAP) to prevent the vector from religating to itself. The BamH1 cut  
10   pSVLGS.1 fragment was then ligated to a 2.4 kb BamH1 to Bgl2 fragment of pEE6hCMV (described in PCT Application No. WO89/01036, also available from Celltech) which was cut with Bgl2, BamH1 and Fsp1 to avoid two fragments of similar size, to yield an 11.2 kb vector designated p6/PSVLGS.1. pSVLGS.1 contains the glutamine synthetase selectable marker gene under control of the SV40 later promoter. The BamH1 to Bgl2 fragment of  
15   pEE6hCMV contains the human cytomegalovirus major immediate early promoter (hCMV), a polylinker, and the SV40 early polyadenylation signal. The coding sequences for soluble TNF-R were added to p6/PSVLGS.1 by excising a Not1 to BamH1 fragment from the expression vector psolTNFR/CAVNOT (made according to Example 3 above), blunt ending with Klenow and ligating with SmaI cut dephosphorylated p6/PSVLGS.1, thereby placing  
20   the solTNF-R coding sequences under the control of the hCMV promoter. This resulted in a single plasmid vector in which the SV40/GS and hCMB/solTNF-R transcription units are transcribed in opposite directions. This vector was designated psolTNFR/P6/PSVLGS.

psolTNFR/P6/PSVLGS was used to transfect CHO-K1 cells (available from ATCC, Rochville, MD, under accession number CCL 61) as follows. A monolayer of CHO-K1 cells  
25   were grown to subconfluency in Minimum Essential Medium (MEM) 10X (Gibco: 330-1581AJ) without glutamine and supplemented with 10% dialysed fetal bovine serum (Gibco: 220-6300AJ), 1 mM sodium pyruvate (Sigma), MEM non-essential amino acids (Gibco: 320-1140AG), 500 µM asparagine and glutamate (Sigma) and nucleosides (30 µM adenosine, guanosine, cytidine and uridine and 10 µM thymidine)(Sigma).

30       Approximately  $1 \times 10^6$  cells per 10 cm petri dish were transfected with 10 ug of psolTNFR/P6/PSVLGS by standard calcium phosphate precipitation, substantially as described by Graham & van der Eb, *Virology* 52:456 (1983). Cells were subjected to glycerol shock (15% glycerol in serum-free culture medium for approximately 1.5 minutes) approximately 4 hours after transfection, substantially as described by Frost & Williams,  
35   *Virology* 91:39 (1978), and then washed with serum-free medium. One day later, transfected cells were fed with fresh selective medium containing MSX at a final concentration of 25 µM. Colonies of MSX-resistant surviving cells were visible within 3-4 weeks. Surviving colonies

were transferred to 24-well plates and allowed to grow to confluency in selective medium. Conditioned medium from confluent wells were then assayed for soluble TNF-R activity using the binding assay described in Example 1 above. These assays indicated that the colonies expressed biologically active soluble TNF-R.

- 5 In order to select for GS gene amplification, several MSX-resistant cell lines are transfected with pSolTNFR/P6/PSVLGS and grown in various concentrations of MSX. For each cell line, approximately  $1 \times 10^6$  cells are plated in gradually increasing concentrations of 100  $\mu$ M, 250  $\mu$ M, 500  $\mu$ M and 1 mM MSX and incubated for 10-14 days. After 12 days, colonies resistant to the higher levels of MSX appear. The surviving colonies are assayed for
- 10 TNF-R activity using the binding assay described above in Example 1. Each of these highly resistant cell lines contains cells which arise from multiple independent amplification events. From these cells lines, one or more of the most highly resistant cells lines are isolated. The amplified cells with high production rates are then cloned by limiting dilution cloning. Mass cell cultures of the transfectants secrete active soluble TNF-R.

15

### Example 8

#### Expression of Soluble Human TNF-R in Yeast

- Soluble human TNF-R was expressed in yeast with the expression vector pIXY432, which was derived from the yeast expression vector pIXY120 and pl. mid pYEP352. pIXY120 is identical to pYaHuGM (ATCC 53157), except that it contains no cDNA insert and includes a polylinker/multiple cloning site with a NcoI restriction site.
- 20

- A DNA fragment encoding TNF receptor and suitable for cloning into the yeast expression vector pIXY120 was first generated by polymerase chain reaction (PCR) amplification of the extracellular portion of the full length receptor from pCAV/NOT-TNF-R
- 25 (ATCC 68088). The following primers were used in this PCR amplification:

#### 5' End Primer

- 30 5' -TTCCGGTACCTTTGGATAAAAGAGACTACAAGGAC  
Asp718->ProLeuAspLysArgAspTyrLysAsp  
GACGATGACAAGTTGCCCGCCAGGTGGCATTTACA-3'  
AspAspAspLys<----- TNF-R ----->

- 35 3' End Primer (antisense)

5' -CCCGGGATCCCTTAGTCGCCAGTGCTCCCTTCAGCTGGG-3'  
BamH1>End<----- TNF-R ----->

The 5' end oligonucleotide primer used in the amplification included an Asp718 restriction site at its 5' end, followed by nucleotides encoding the 3' end of the yeast  $\alpha$ -factor leader sequence (Pro-Leu-Asp-Lys-Arg) and those encoding the 8 amino acids of the FLAG<sup>®</sup> peptide (AspTyrLysAspAspAspAspLys) fused to sequence encoding the 5' end of the mature receptor. The FLAG<sup>®</sup> peptide (Hopp et al., *Bio/Technology* 6:1204, 1988) is a highly antigenic sequence which reversibly binds the monoclonal antibody M1 (ATCC HB 9259). The oligonucleotide used to generate the 3' end of the PCR-derived fragment is the *antisense* strand of DNA encoding sequences which terminate the open reading frame of the receptor after nucleotide 704 of the mature coding region (following the Asp residue preceding the transmembrane domain) by introducing a TAA stop codon (underlined). The stop codon is then followed by a BamH1 restriction site. The DNA sequences encoding TNF-R are then amplified by PCR, substantially as described by Innis et al., eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, 1990).

The PCR-derived DNA fragment encoding soluble human TNF-R was subcloned into the yeast expression vector pIXY120 by digesting the PCR-derived DNA fragment with BamH1 and Asp718 restriction enzymes, digesting pIXY120 with BamH1 and Asp718, and ligating the PCR fragment into the cut vector *in vitro* with T4 DNA ligase. The resulting construction (pIXY424) fused the open reading frame of the FLAG<sup>®</sup>-soluble TNF receptor in-frame to the complete  $\alpha$ -factor leader sequence and placed expression in yeast under the aegis of the regulated yeast alcohol dehydrogenase (ADH2) promoter. Identity of the nucleotide sequence of the soluble TNF receptor carried in pIXY424 with those in cDNA clone 1 were verified by DNA sequencing using the dideoxynucleotide chain termination method. pIXY424 was then transformed into *E. coli* strain RR1.

Soluble human TNF receptor was also expressed and secreted in yeast in a second vector. This second vector was generated by recovering the pIXY424 plasmid from *E. coli* and digesting with EcoR1 and BamH1 restriction enzymes to isolate the fragment spanning the region encoding the ADH2 promoter, the  $\alpha$ -factor leader, the FLAG<sup>®</sup>-soluble TNF receptor and the stop codon. This fragment was ligated *in vitro* into EcoR1 and BamH1 cut plasmid pYEP352 (Hill et al., *Yeast* 2:163 (1986)), to yield the expression plasmid pIXY432, which was transformed into *E. coli* strain RR1.

To assess secretion of the soluble human TNF receptor from yeast, pIXY424 was purified and introduced into a diploid yeast strain of *S. cerevisiae* (XV2181) by electroporation and selection for acquisition of the plasmid-borne yeast TRP1<sup>+</sup> gene on media lacking tryptophan. To assess secretion of the receptor directed by pIXY432, the plasmid was introduced into the yeast strain PB149-6b by electroporation followed by selection for the plasmid-borne URA3<sup>+</sup> gene with growth on media lacking uracil. Overnight cultures were grown at 30°C in the appropriate selective media. The PB149-6b/pIXY434

transformants were diluted into YEP-1% glucose media and grown at 30°C for 38-40 hours. Supernatants were prepared by removal of cells by centrifugation, and filtration of supernatants through 0.45µ filters.

The level of secreted receptor in the supernatants was determined by immuno-dotblot. Briefly, 1 ul of supernatants, and dilutions of the supernatants, were spotted onto nitrocellulose filters and allowed to dry. After blocking non-specific protein binding with a 3% BSA solution, the filters were incubated with diluted M1 anti-FLAG® antibody, excess antibody was removed by washing and then dilutions of horseradish peroxidase conjugated anti-mouse IgG antibodies were incubated with the filters. After removal of excess secondary antibodies, peroxidase substrates were added and color development was allowed to proceed for approximately 10 minutes prior to removal of the substrate solution.

The anti-FLAG® reactive material found in the supernatants demonstrated that significant levels of receptor were secreted by both expression systems. Comparisons demonstrated that the pIXY432 system secreted approximately 8-16 times more soluble human TNF receptor than the pIXY424 system. The supernatants were assayed for soluble TNF-R activity, as described in Example 1, by their ability to bind <sup>125</sup>I-TNFα and block TNFα-binding. The pIXY432 supernatants were found to contain significant levels of active soluble TNF-R.

20

### Example 9

#### Isolation of Murine TNF-R cDNAs

Murine TNF-R cDNAs were isolated from a cDNA library made from murine 7B9 cells, an antigen-dependent helper T cell line derived from C57BL/6 mice, by cross-species hybridization with a human TNF-R probe. The cDNA library was constructed in λZAP (Stratagene, San Diego), substantially as described above in Example 2, by isolating polyadenylated RNA from the 7B9 cells.

A double-stranded human TNF-R cDNA probe was produced by excising an approximately 3.5 kb Not1 fragment of the human TNF-R clone 1 and <sup>32</sup>P-labeling the cDNA using random primers (Boehringer-Mannheim).

The murine cDNA library was amplified once and a total of 900,000 plaques were screened, substantially as described in Example 2, with the human TNF-R cDNA probe. Approximately 21 positive plaques were purified, and the Bluescript plasmids containing EcoR1-linkered inserts were excised (Stratagene, San Diego). Nucleic acid sequencing of a portion of murine TNF-R clone 11 indicated that the coding sequence of the murine TNF-R was approximately 88% homologous to the corresponding nucleotide sequence of human



TNF-R. A partial nucleotide sequence of murine TNF-R cDNA clone 11 is set forth in Figures 4-5.

### Example 10

#### 5                    Preparation of Monoclonal Antibodies to TNF-R

Preparations of purified recombinant TNF-R, for example, human TNF-R, or transfected COS cells expressing high levels of TNF-R are employed to generate monoclonal antibodies against TNF-R using conventional techniques, for example, those disclosed in  
10 U.S. Patent 4,411,993. Such antibodies are likely to be useful in interfering with TNF binding to TNF receptors, for example, in ameliorating toxic or other undesired effects of TNF, or as components of diagnostic or research assays for TNF or soluble TNF receptor.

To immunize mice, TNF-R immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice. Ten to  
15 twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an  
20 appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell  
25 hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with TNF-R, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Patent 4,703,004. Positive clones are then injected into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations  
30 (>1 mg/ml) of anti-TNF-R monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

**CLAIMS**

1. An isolated DNA sequence encoding a biologically active mammalian TNF  
5 receptor (TNF-R) protein.
2. An isolated DNA sequence according to claim 1, selected from the group  
consisting of:
  - (a) cDNA clones having a nucleotide sequence derived from the coding region of a  
10 native mammalian TNF-R gene;
  - (b) DNA sequences capable of hybridization to the clones of (a) under moderately  
stringent conditions (50°C, 2 x SSC) and which encode biologically active TNF-R protein;  
and
  - (c) DNA sequences which are degenerate as a result of the genetic code to the DNA  
15 sequences defined in (a) and (b) and which encode biologically active TNF-R protein.
3. An isolated DNA sequence according to claim 1 which encodes a soluble human  
TNF-R protein.
- 20 4. An isolated DNA sequence according to claim 3, wherein the soluble human  
TNF-R protein has an amino acid sequence comprises the sequence of amino acid residues 1-  
x of Figure 2, wherein x is selected from the group consisting of amino acids 163-235
5. An isolated DNA sequence according to claim 3, wherein the soluble human  
25 TNF-R protein comprises the sequence of amino acids 1-235 of Figure 2.
6. A DNA sequence according to claim 5, wherein amino acid residue 46 is selected  
from the group consisting of Ile and Thr and amino acid residue 118 is selected from the  
group consisting of Val and Ile.  
30
7. An isolated DNA sequence according to claim 3, wherein the soluble human  
TNF-R protein comprises the sequence of amino acids 1-185 of Figure 2.
8. An isolated DNA sequence according to claim 3, wherein the soluble human  
35 TNF-R protein comprises the sequence of amino acids 1-163 of Figure 2.
9. A recombinant expression vector comprising a DNA sequence according to any  
one of claims 1-8.

10. A process for preparing a biologically active mammalian TNF receptor (TNF-R) protein, comprising culturing a suitable host cell comprising a vector according to claim 8 under conditions promoting expression.
- 5 11. A purified biologically active mammalian TNF receptor (TNF-R) protein composition.
12. A purified biologically active soluble human TNF-R protein composition.
- 10 13. A purified biologically active TNF-R protein composition according to claim 12, comprising the sequence of amino acid residues 1-235 of Figure 2.
- 15 14. A purified biologically active TNF-R protein composition according to claim 12, comprising the sequence of amino acid residues 1-185 of Figure 2.
- 15 15. A purified biologically active TNF-R protein composition according to claim 12, comprising the sequence of amino acid residues 1-163 of Figure 2.
- 20 16. A composition for regulating immune responses in a mammal, comprising an effective amount of a mammalian TNF-R protein composition according to claim 11, and a suitable diluent or carrier.
- 25 17. A method for regulating immune responses in a mammal, comprising administering an effective amount of a composition according to claim 16.
18. The method of claim 17, wherein the TNF-R protein is human TNF-R and the mammal to be treated is a human.
- 30 19. The use of mammalian TNF-R protein in preparing a pharmaceutical composition suitable for parenteral administration to a human patient for regulating immune responses.
20. An assay method for detection of TNF or TNF-R molecules or the interaction thereof, comprising use of a protein composition according to claim 11.
- 35 21. Antibodies immunoreactive with mammalian TNF receptors.

## AMENDED CLAIMS

[received by the International Bureau  
on 20 February 1991 (20.02.91);  
original claim 1 amended;  
other claims unchanged (1 page)]

1. (Amended) An isolated DNA sequence encoding a biologically active mammalian TNF receptor (TNF-R) protein whose mature form has a molecular weight of about 80 kilodaltons.  
5
2. An isolated DNA sequence according to claim 1, selected from the group consisting of:
  - (a) cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian TNF-R gene;  
10
  - (b) DNA sequences capable of hybridization to the clones of (a) under moderately stringent conditions (50°C, 2 x SSC) and which encode biologically active TNF-R protein; and
  - (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active TNF-R protein.  
15
3. An isolated DNA sequence according to claim 1 which encodes a soluble human TNF-R protein.  
20
4. An isolated DNA sequence according to claim 3, wherein the soluble human TNF-R protein has an amino acid sequence comprising the sequence of amino acids 1-x of Figure 2, wherein x is selected from the group consisting of amino acids 163-235.
- 25 5. An isolated DNA sequence according to claim 3, wherein the soluble human TNF-R protein comprises the sequence of amino acids 1-235 of Figure 2..
6. A DNA sequence according to claim 5, wherein amino acid residue 46 is selected from the group consisting of Ile and Thr and amino acid residue 118 is selected from the group consisting of Val and Ile.  
30
7. An isolated DNA sequence according to claim 3, wherein the soluble human TNF-R protein has an amino acid sequence comprising the sequence of amino acids 1-x of Figure 2, wherein x is selected from the group consisting of amino acids 163-235.  
35
8. An isolated DNA sequence according to claim 3, wherein the soluble human TNF-R protein comprises the sequence of amino acids 1-163 of Figure 2.
9. A recombinant expression vector comprising a DNA sequence according to any one of claims 1-8.  
40

**STATEMENT UNDER ARTICLE 19**

Pursuant to PCT Article 19, Applicant has amended Claim 1 of the above-referenced patent application to include the additional limitation that the mature form of the claimed TNF receptor has a molecular weight of about 80 kilodaltons.

The Amendment distinguishes the claimed TNF receptor from the TNF receptor disclosed by EP 0308378 (Yeda Research and Development Co. Ltd.), which is a different TNF receptor protein, having a molecular weight of about 60 kilodaltons. Applicant's TNF receptor is further distinguishable by the fact that its human form has a mature N-terminal amino acid sequence of Leu-Pro-Ala-Gln-Val; in contrast, the human mature N-terminal amino acid sequence of the Yeda TNF receptor is Asp-Ser-Val-Cys-Pro. Further comparison of amino acid sequence confirms that the receptors are distinct.

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Figure 1



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Figure 2

GCGAGGCAGGCAGCCTGGAGAGAAGGCG															28
CTGGGCTGCGAGGGCGCGAGGGCGCGAGGGCAGGGGGCAACCGGACCCCGCCCGCATCC															87
ATG	GCG	CCC	GTC	GCC	GTC	TGG	GCC	GCG	CTG	GCC	GTC	GGA	CTG	GAG	132
Met	Ala	Pro	Val	Ala	Val	Trp	Ala	Ala	Leu	Ala	Val	Gly	Leu	Glu	-8
CTC	TGG	GCT	GCG	GCG	CAC	GCC	TTG	CCC	GCC	CAG	GTG	GCA	TTT	ACA	177
Leu	Trp	Ala	Ala	Ala	His	Ala	<u>Leu</u>	Pro	Ala	Gln	Val	Ala	Phe	Thr	8
CCC	TAC	GCC	CCG	GAG	CCC	GGG	AGC	ACA	TGC	CGG	CTC	AGA	GAA	TAC	222
Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	23
TAT	GAC	CAG	ACA	GCT	CAG	ATG	TGC	TGC	AGC	AAA	TGC	TCG	CCG	GGC	267
Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	Lys	Cys	Ser	Pro	Gly	38
CAA	CAT	GCA	AAA	GTC	TTC	TGT	ACC	AAG	ACC	TCG	GAC	ACC	GTG	TGT	312
Gln	His	Ala	Lys	Val	Phe	Cys	Thr	Lys	Thr	Ser	Asp	Thr	Val	Cys	53
GAC	TCC	TGT	GAG	GAC	AGC	ACA	TAC	ACC	CAG	CTC	TGG	AAC	TGG	GTT	357
Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	Asn	Trp	Val	68
CCC	GAG	TGC	TTG	AGC	TGT	GGC	TCC	CGC	TGT	AGC	TCT	GAC	CAG	GTG	402
Pro	Glu	Cys	Leu	Ser	Cys	Gly	Ser	Arg	Cys	Ser	Ser	Asp	Gln	Val	83
GAA	ACT	CAA	GCC	TGC	ACT	CGG	GAA	CAG	AAC	CGC	ATC	TGC	ACC	TGC	447
Glu	Thr	Gln	Ala	Cys	Thr	Arg	Glu	Gln	Asn	Arg	Ile	Cys	Thr	Cys	98
AGG	CCC	GGC	TGG	TAC	TGC	GCG	CTG	AGC	AAG	CAG	GAG	GGG	TGC	CGG	492
Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys	Gln	Glu	Gly	Cys	Arg	113
CTG	TGC	GCG	CCG	CTG	CGC	AAG	TGC	CGC	CCG	GGC	TTC	GGC	GTG	GCC	537
Leu	Cys	Ala	Pro	Leu	Arg	Lys	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	128
AGA	CCA	GGA	ACT	GAA	ACA	TCA	GAC	GTG	GTG	TGC	AAG	CCC	TGT	GCC	582
Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	143
CCG	GGG	ACG	TTC	TCC	AAC	ACG	ACT	TCA	TCC	ACG	GAT	ATT	TGC	AGG	627
Pro	Gly	Thr	Phe	Ser	Asn	Thr	Thr	Ser	Ser	Thr	Asp	Ile	Cys	Arg	158
CCC	CAC	CAG	ATC	TGT	AAC	GTG	GTG	GCC	ATC	CCT	GGG	AAT	GCA	AGC	672
Pro	His	Gln	Ile	Cys	Asn	Val	Val	Ala	Ile	Pro	Gly	Asn	Ala	Ser	173
ATG	GAT	GCA	GTC	TGC	ACG	TCC	ACG	TCC	CCC	ACC	CGG	AGT	ATG	GCC	717
Met	Asp	Ala	Val	Cys	Thr	Ser	Thr	Ser	Pro	Thr	Arg	Ser	Met	Ala	188
CCA	GGG	GCA	GTA	CAC	TTA	CCC	CAG	CCA	GTG	TCC	ACA	CGA	TCC	CAA	762
Pro	Gly	Ala	Val	His	Leu	Pro	Gln	Pro	Val	Ser	Thr	Arg	Ser	Gln	203
CAC	ACG	CAG	CCA	ACT	CCA	GAA	CCC	AGC	ACT	GCT	CCA	AGC	ACC	TCC	807
His	Thr	Gln	Pro	Thr	Pro	Glu	Pro	Ser	Thr	Ala	Pro	Ser	Thr	Ser	218
TTC	CTG	CTC	CCA	ATG	GGC	CCC	AGC	CCC	CCA	GCT	GAA	GGG	AGC	ACT	852
Phe	L u	Leu	Pro	Met	Gly	Pro	Ser	Pro	Pro	Ala	Glu	Gly	Ser	Thr	233
GGC	GAC	TTC	GCT	CTT	CCA	GTT	GGA	CTG	ATT	GTG	GGT	GTG	ACA	GCC	897
Gly	Asp	<u>Phe</u>	<u>Ala</u>	<u>Leu</u>	<u>Pro</u>	<u>Val</u>	<u>Gly</u>	<u>Leu</u>	<u>Ile</u>	<u>Val</u>	<u>Gly</u>	<u>Val</u>	<u>Thr</u>	<u>Ala</u>	248

SUBSTITUTE SHEET

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Figure 3

TTG GGT CTA CTA ATA ATA GGA GTG GTG AAC TGT GTC ATC ATG ACC	942
<u>Leu Gly Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr</u>	263
CAG GTG AAA AAG AAG CCC TTG TGC CTG CAG AGA GAA GCC AAG GTG	987
<u>Gln Val Lys Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val</u>	278
CCT CAC TTG CCT GCC GAT AAG GCC CGG GGT ACA CAG GGC CCC GAG	1032
<u>Pro His Leu Pro Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu</u>	293
CAG CAG CAC CTG CTG ATC ACA GCG CCG AGC TCC AGC AGC AGC TCC	1077
<u>Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser</u>	308
CTG GAG AGC TCG GCC AGT GCG TTG GAC AGA AGG GCG CCC ACT CGG	1122
<u>Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala Pro Thr Arg</u>	323
AAC CAG CCA CAG GCA CCA GGC GTG GAG GCC AGT GGG GCC GGG GAG	1167
<u>Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu</u>	338
GCC CGG GCC AGC ACC GGG AGC TCA GAT TCT TCC CCT GGT GGC CAT	1212
<u>Ala Arg Ala Ser Thr Gly Ser Ser Asp Ser Ser Pro Gly Gly His</u>	353
GGG ACC CAG GTC AAT GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC	1257
<u>Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser Ser</u>	368
TCT GAC CAC AGC TCA CAG TGC TCC TCC CAA GCC AGC TCC ACA ATG	1302
<u>Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met</u>	383
GGA GAC ACA GAT TCC AGC CCC TCG GAG TCC CCG AAG GAC GAG CAG	1347
<u>Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln</u>	398
GTC CCC TTC TCC AAG GAG GAA TGT GCC TTT CGG TCA CAG CTG GAG	1392
<u>Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu</u>	413
ACG CCA GAG ACC CTG CTG GGG AGC ACC GAA GAG AAG CCC CTG CCC	1437
<u>Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro</u>	428
CTT GGA GTG CCT GAT GCT GGG ATG AAG CCC AGT	1470
<u>Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser</u>	439

TAACCAGGCCGGTGTGGGCTGTGTCGTAGCCAAGGTGGGCTGAGCCCTGGCAGGATGAC

CCTGCGAAGGGGCCCTGGTCCTTCCAGGCCCCCACCCTAGGACTCTGAGGCTCTTTCT

GGGCCAAGTTCTCTAGTGCCCTCCACAGCCGCAGCCTCCCTCTGACCTGCAG



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**Figure 4**

CGCAGCTGAGGCACTAGAGCTCC															23
AGGCACAAGGGCGGGAGCCACCGCTGCCCCCT															75
	Met	Ala	Pro	Ala	Ala	Leu	Trp								-16
GTC GCG CTG GTC TTC GAA CTG CAG CTG TGG GCC ACC GGG CAC ACA															120
Val Ala Leu Val Phe Glu Leu Gln Leu Trp Ala Thr Gly His Thr															-1
GTG CCC GCC CAG GTT GTC TTG ACA CCC TAC AAA CCG GAA CCT GGG															165
<u>Val</u> Pro Ala Gln Val Val Leu Thr Pro Tyr Lys Pro Glu Pro Gly															15
TAC GAG TGC CAG ATC TCA CAG GAA TAC TAT GAC AGG AAG GCT CAG															210
Tyr Glu Cys Gln Ile Ser Gln Glu Tyr Tyr Asp Arg Lys Ala Gln															30
ATG TGC TGT GCT AAG TGT CCT CCT GGC CAA TAT GTG AAA CAT TTC															255
Met Cys Cys Ala Lys Cys Pro Pro Gly Gln Tyr Val Lys His Phe															45
TGC AAC AAG ACC TCG GAC ACC GTG TGT GCG GAC TGT GAG GCA AGC															300
Cys Asn Lys Thr Ser Asp Thr Val Cys Ala Asp Cys Glu Ala Ser															60
ATG TAT ACC CAG GTC TGG AAC CAG TTT CGT ACA TGT TTG AGC TGC															345
Met Tyr Thr Gln Val Trp Asn Gln Phe Arg Thr Cys Leu Ser Cys															75
AGT TCT TCC TGT ACC ACT GAC CAG GTG GAG ATC CGC GCC TGC ACT															390
Ser Ser Ser Cys Thr Thr Asp Gln Val Glu Ile Arg Ala Cys Thr															90
AAA CAG CAG AAC CGA GTG TGT GCT TGC GAA GCT GGC AGG TAC TgC															435
Lys Gln Gln Asn Arg Val Cys Ala Cys Glu Ala Gly Arg Tyr Cys															105
GCC TTG AAA ACC CAT TCT GGC AGC TGT CGA CAG TGC ATG AGG CTG															480
Ala Leu Lys Thr His Ser Gly Ser Cys Arg Gln Cys Met Arg Leu															120
AGC AAG TGC GGC CCT GGC TTC GGA GTG GCC AGT TCA AGA GCC CCA															525
Ser Lys Cys Gly Pro Gly Phe Gly Val Ala Ser Ser Arg Ala Pro															135
AAT GGA AAT GTG CTA TGC AAG GCC TGT GCC CCA GGG ACG TTC TCT															570
Asn Gly Asn Val Leu Cys Lys Ala Cys Ala Pro Gly Thr Phe Ser															150
GAC ACC ACA TCA TCC ACT GAT GTG TGC AGG CCC CAC CGC ATC TGT															615
Asp Thr Thr Ser Ser Thr Asp Val Cys Arg Pro His Arg Ile Cys															165
AGC ATC CTG GCT ATT CCC GGA AAT GCA AGC ACA GAT GCA GTC TGT															660
Ser Ile Leu Ala Ile Pro Gly Asn Ala Ser Thr Asp Ala Val Cys															180
GCG CCC GAG TCC CCA ACT CTA AGT GCC ATC CCA AGG ACA CTC TAC															705
Ala Pro Glu Ser Pro Thr Leu Ser Ala Ile Pro Arg Thr Leu Tyr															195
GTA TCT CAG CCA GAG CCC ACA AGA TCC CAA CCC CTG GAT CAA GAG															750
Val Ser Gln Pro Glu Pro Thr Arg Ser Gln Pro Leu Asp Gln Glu															210
CCA GGG CCC AGC CAA ACT CCA AGC ATC CTT ACA TCG TTG GGT TCA															795
Pro Gly Pro Ser Gln Thr Pro Ser Ile Leu Thr Ser Leu Gly Ser															225
ACC CCC ATT ATT GAA CAA AGT ACC AAG GGT GGC ATC TCT CTT CCA															840
Thr Pro Ile Ile Glu Gln Ser Thr <u>Lys Gly Gly Ile Ser Leu Pro</u>															240

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Figure 5

ATT GGT CTG ATT GTT GGA GTG ACA TCA CTG GGT CTG CTG ATG TTA	885
<u>Ile Gly Leu Ile Val Gly Val Thr Ser Leu Gly Leu Leu Met Leu</u>	255
GGA CTG GTG AAC TGC ATC ATC CTG GTG CAG AGG AAA AAG AAG CCC	930
<u>Gly Leu Val Asn Cys Ile Ile Leu Val Gln Arg Lys Lys Lys Pro</u>	270
TCC TGC CTA CAA AGA GAT GCC AAG GTG CCT CAT GTG CCT GAT GAG	975
<u>Ser Cys Leu Gln Arg Asp Ala Lys Val Pro His Val Pro Asp Glu</u>	285
AAA TCC CAG GAT GCA GTA GGC CTT GAG CAG CAG CAC CTG TTG ACC	1020
<u>Lys Ser Gln Asp Ala Val Gly Leu Glu Gln Gln His Leu Leu Thr</u>	300
ACA GCA CCC AGT TCC AGC AGC AGC TCC CTA GAG AGC TCA GCC AGC	1065
<u>Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser</u>	315
GCT GGG GAC CGA AGG GCG CCC CCT GGG GGC CAT CCC CAA GCA AGA	1110
<u>Ala Gly Asp Arg Arg Ala Pro Pro Gly Gly His Pro Gln Ala Arg</u>	330
GTC ATG GCG GAG GCC CAA GGG TTT CAG GAG GCC CGT GCC AGC TCC	1155
<u>Val Met Ala Glu Ala Gln Gly Phe Gln Glu Ala Arg Ala Ser Ser</u>	345
AGG ATT TCA GAT TCT TCC CAC GGA AGC CAC GGG ACC CAC GTC AAC	1200
<u>Arg Ile Ser Asp Ser Ser His Gly Ser His Gly Thr His Val Asn</u>	360
GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC TCT GAC CAC AGT TCT	1245
<u>Val Thr Cys Ile Val Asn Val Cys Ser Ser Ser Asp His Ser Ser</u>	375
CAG TGC TCT TCC CAA GCC AGC GCC ACA GTG GGA GAC CCA GAT GCC	1290
<u>Gln Cys Ser Ser Gln Ala Ser Ala Thr Val Gly Asp Pro Asp Ala</u>	390
AAG CCC TCA GCG TCC CCA AAG GAT GAG CAG GTC CCC TTC TCT CAG	1335
<u>Lys Pro Ser Ala Ser Pro Lys Asp Glu Gln Val Pro Phe Ser Gln</u>	405
GAG GAG TGT CCG TCT CAG TCC CCG TGT GAG ACT ACA GAG ACA CTG	1380
<u>Glu Glu Cys Pro Ser Gln Ser Pro Cys Glu Thr Thr Glu Thr Leu</u>	420
CAG AGC CAT GAG AAG CCC TTG CCC CTT GGT GTG CCG GAT ATG GGC	1425
<u>Gln Ser His Glu Lys Pro Leu Pro Leu Gly Val Pro Asp Met Gly</u>	435
ATG AAG CCC AGC CAA GCT GGC TGG TTT GAT CAG ATT GCA GTC AAA	1470
<u>Met Lys Pro Ser Gln Ala Gly Trp Phe Asp Gln Ile Ala Val Lys</u>	450
GTG GCC	1476
<u>Val Ala</u>	452

TGACCCCTGACAGGGGTAACACCCTGCAAAGGGACCCCCGAGACCCTGAACCCATGGAAC	1536
TTCATGACTTTTGCTGGATCCATTTCCTTAGTGGCTTCCAGAGCCCCAGTTGCAGGTCA	1596
AGTGAGGGCTGAGACAGCTAGAGTGGTCAAAAAGTCCATGGTGTGTTTATGGGGGCAGTC	1656
CCAGGAAGTTGTTGCTCTTCCATGACCCCTCTGGATCTCCTGGGCTCTTGCTGATTCTT	1716
GCTTCTGAGAGGCCCCAGTATTTTTTCCTTCTAAGGAGCTAACATCCTCTTCCATGAATA	1776
GCACAGCTCTTCAGCCTGAATGCTGACACTGCAGGGCGGTTCCAGCAAGTAGGAGCAAGT	1836
GGTGGCCTGGTAGGGCACAGAGGCCCTTCAGGTTAGTGCTAAACTCTTAGGAAGTACCCT	1896
CTCCAAGCCCACCGAAATTCTTTTGATGCAAGAATCAGAGGCCCATCAGGCAGAGTTGC	1956

**Figure 6**

**SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 90/04001

<b>I. CLASSIFICATION F SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 12 N 15/12, C 12 P 21/02, A 61 K 37/02, C 12 P 21/08, G 01 N 33/68		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System <sup>1</sup>	Classification Symbols	
IPC <sup>5</sup>	C 12 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
P,X	Science, vol. 248, 25 May 1990, (Washington, US) C.A. Smith et al.: "A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins", pages 1019-1023, see the whole article --	1-10
P,X	Proc. Natl. Acad. Sci, US, vol. 87, August 1990 (Washington, DC, US) K.A. Heller et al.: "Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor", pages 6151-6155, see the whole article --	1-8, 11
P,X	Cell, vol. 61, 20 April 1990, Cell Press (Cambridge, Mass. US) H. Loetscher et al.: "Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor", pages 351-359, see the whole article (cited in the application)	1, 2, 9-11
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th November 1990	20 DEC 1990	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Mme N. KUIPER	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Cell, vol. 61, 20 April 1990, Cell Press, (Cambridge, Mass. US) T.J. Schall et al.: "Molecular cloning and expression of a receptor for human tumor necrosis factor", pages 361-370 see the whole article  (cited in the application) --	1,2,9-11
P,X	The Journal of Biological Chemistry, vol. 265, no. 3, 25 January 1990 (Baltimore, US) H. Engelmann et al.: "Two tumor necrosis factor-binding proteins purified from human urine" pages 1531-1536, see the whole article  (cited in the application) --	11,12,21
P,X	GB, A, 2218101 (GLAXO GROUP LTD) 8 November 1989, see claims  (cited in the application) --	1-3,9-12, 16,19
X	EP, A, 0308378 (YEDA RESEARCH AND DEVELOPMENT CO. LTD) 22 March 1989, see claims --	1-3,9-12, 16,19,21
P,X	EP, A, 0334165 (HOFFMANN-LA ROCHE AG) 27 September 1989, see claims; page 4, example 1  -----	11,21

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers .....\*..... because they relate to subject matter not required to be searched by this Authority, namely:

\*17-18

See PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9004001  
SA 39605

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/12/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 2218101	08-11-89	AU-A- 3228789	05-10-89
		BE-A- 1001845	20-03-90
		FR-A- 2629345	06-10-89
		JP-A- 2117700	02-05-90
		NL-A- 8900779	16-10-89
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		JP-A- 2000200	05-01-90
EP-A- 0334165	27-09-89	AU-A- 3125289	21-09-89
		JP-A- 2154695	14-06-90